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EFFECTS OF RAPID ANTIGEN DEGRADATION AND VEE GLYCOPROTEIN SPECIFICITY ON IMMUNE RESPONSES INDUCED BY A VEE REPLICON VACCINE

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Abstract

Genetic vaccines are engineered to produce immunogens de novo in the cells of the host for stimulation of a protective immune response. In some of these systems, antigens engineered for rapid degradation have produced an enhanced cellular immune response by more efficient entry into pathways for processing and presentation of MHC class I peptides. VEE replicon particles (VRP), single cycle vaccine vectors derived from Venezuelan equine encephalitis virus (VEE), are examined here for the effect of an increased rate of immunogen degradation on VRP vaccine efficacy. VRP expressing the matrix capsid (MA/CA) portion of SIV Gag were altered to promote rapid degradation of MA/CA by various linkages to co-translated ubiquitin or by destabilizing mutations and were used to immunize BALB/c mice for quantitation of the anti-MA/CA cellular and humoral immune responses. Rapid degradation by the N-end rule correlated with a dampened immune response relative to unmodified MA/CA when the VRP carried a glycoprotein spike from an attenuated strain of VEE. In contrast, statistically equivalent numbers of IFNγ^+ T-cells resulted when VRP expressing unstable MA/CA were packaged with the wild-type VEE glycoproteins. These results suggest that the cell types targeted *in vivo* by VRP carrying mutant or wild type glycoprotein spikes are functionally different, and are consistent with previous findings suggesting that wild-type VEE glycoproteins preferentially target professional antigen presenting cells that use peptides generated from the degraded antigen for direct presentation on MHC.

INTRODUCTION

Many agents of human disease remain serious threats in the absence of effective and affordable means of control or treatment. However, there now exist powerful tools for identifying and

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altering the proteins of these disease agents, as well as for developing delivery systems to enhance their immunogenicity and safety. New generation genetic vaccines, that is DNA plasmids or vaccine vectors that deliver genes rather than proteins to the host, produce immunogens inside the host's own cells for stimulation of a protective immune response. These vaccines combine the safety features of subunit vaccines, which carry immunogenic but not pathogenic determinants of the disease agent, with the ability of a live virus vaccine to present de novo synthesized proteins to the host immune system. This is especially important for the stimulation of a cellular immune response, in that newly synthesized proteins can be processed for presentation on the host MHC complexes in an authentic context.

Important insights into the mechanism of action of vaccine vectors can be gained through the use of different forms of antigen. For example, antigens designed for more rapid degradation may elicit an enhanced cellular immune response by more efficient entry into pathways for processing and presentation of MHC class I peptides in a professional antigen presenting cell (APC). However, the effect of decreased protein stability may not have this effect for vectors that target different cells of the host immune system. Several studies of antigens targeted for more rapid proteasomal degradation have found that delivery of rapidly degraded antigens stimulates an enhanced cellular immune response *in vivo* when delivered by DNA vaccination (Andersson and Barry, 2004; Delogu et al., 2000; Duan et al., 2006; Rodriguez et al., 1998; Rodriguez, Zhang, and Whitton, 1997; Wu and Kipps, 1997) or by recombinant vaccinia virus (Tobery and Siliciano, 1997; Townsend et al., 1988). As suggested previously by several groups (Andersson and Barry, 2004; Huckriede et al., 2004; Whitton et al., 1999), more efficient processing and presentation of an antigen would be predicted to increase its ability to induce a cellular immune response if the vector targets a professional APC, and if production of appropriately processed peptides from the unaltered antigen is the limiting step in immune induction. If, however, the vector were to target a non-APC and induce immunity by cross presentation, rapid antigen degradation would be predicted to be unfavorable. In a series of adoptive transfer experiments in mice, it was shown that the transfer of intact proteins, not peptides, is optimal for cross presentation when the antigen is expressed in a non-APC (Norbury et al., 2004).

An increased rate of protein degradation can be achieved by several different strategies including ubiquitination, the N-end rule, and mutations that affect protein folding. In the normal proteasomal degradation pathway, proteins are targeted for degradation by the 26S proteasome through the covalent attachment of ubiquitin (Ub) to an internal lysine residue in the target protein. This first step can be simulated artificially by engineering a protein at the level of the gene sequence to carry a co-translated, non-removable N-terminal or internal Ub monomer, thereby greatly increasing the efficiency of entry into the degradation pathway. Alternatively, the N-end rule describes the correlation between the half-life of a protein and the identity of its N-terminal amino acid (Varshavsky, 1992). Certain N-terminal amino acids, especially those with bulky or charged side-chains, are destabilizing. The function of the N-end rule in targeting a protein for more rapid degradation can be utilized by engineering a protein to have a removable Ub moiety at the N-terminus, which upon cleavage exposes the selected N-terminal residue of the protein. Finally, an increased rate of protein degradation can also be achieved by modifications which cause a protein to fold improperly. The inherently high rate of misfolding which occurs during protein synthesis makes it necessary for the cell to eliminate these misfolded proteins to prevent the build-up of toxic aggregates. This is accomplished primarily by proteasomal degradation (Esser, Alberti, and Hohfeld, 2004; Murata et al., 2001). Strategies to induce protein misfolding in order to target the protein for rapid degradation include sequence changes or rearrangements that disrupt critical structural elements (Wong et al., 2004).

A vaccine vector has been constructed based on the alphavirus, Venezuelan equine encephalitis virus (VEE). The VEE replicon particle (VRP) is a defective virus particle with a self-replicating RNA genome lacking the structural protein genes of the virus. VRP are packaged *in trans* using helper RNAs that express VEE structural proteins, can program only a single cycle of replication, and are unable to produce progeny virus particles (Pushko et al., 1997). Studies in mice showed that soon after inoculation VRP are found in the draining lymph node, where they produce high levels of the heterologous protein whose gene they carry (Grieder et al., 1995; MacDonald and Johnston, 2000). VRP packaged with the wild type V3000 glycoproteins (VRP-3000) infect Langerhans-like cells at the site of inoculation that then move rapidly to the draining lymph node where they express surface markers found on APCs. Specific mutations in the glycoprotein genes of the virus alter the early steps in virus spread, probably due to changes in the efficiency of infection of the Langerhans-like cell. The V3014 glycoprotein spike carries two mutations that attenuate VEE virulence, but still allow efficient packaging of VRP (Grieder et al., 1995; Pushko et al., 1997). VRP carrying the V3014 spike as an added safety feature (VRP-3014) have been used in several preclinical studies and in a human Phase I trial (Chulay, 2006). In continuing studies of VRP cell targeting *in vivo*, preliminary results showed VRP-3014 primarily in cells that did not exhibit the dendritic cell morphology or the localization in the lymph node of the cell target for VRP-3000 (West, Johnson, and Johnston, unpublished results).

To further characterize the VRP vector system the effect of antigen stability was examined using VRP packaged with wild type or mutant glycoproteins. VRP expressing the matrix capsid (MA/CA) portion of SIV Gag were altered to promote rapid degradation of MA/CA by various linkages to co-translated Ub or by destabilizing mutations, and were used to immunize BALB/c mice for quantitation of the anti-MA/CA cellular and humoral immune responses.

MATERIALS AND METHODS

2.1. Cells

Baby hamster kidney (BHK-21) cells were maintained in 1X MEM-α (Minimal Essential Medium Alpha), supplemented with 10% Donor Calf serum (DCS), 10% tryptose phosphate broth, 2 mM L-Glutamine and 100 U/ml each penicillin and streptomycin. Cultures were maintained at 37°C in a CO_2 incubator.

2.2. Plasmids

A mutant mouse Ub sequence, obtained from pCMV-U-MG34, the kind gift of Lindsay Whitton (Rodriguez et al., 1998), was amplified by PCR using primers designed to add a unique ClaI restriction site upstream of the initiating ATG, as well as preserve a unique EcoRI restriction site downstream of the Ala codon 76. PCR was also used to amplify the SIVsm H-4 Gag gene from a VEE replicon plasmid analogous to that for SIVsm H-4i matrix-capsid (MA/CA) described previously (Davis et al., 2000), using primers designed to add an EcoRI restriction site upstream of the initial ATG of Gag, and a ClaI site downstream of the Gag stop codon. This Gag gene carried a change from Gly to Ala in codon 2 to ablate the myristylation signal (Davis et al., 2000). In a third PCR reaction (overlap reaction) the two PCR products were linked together to form a DNA fragment with a single open reading frame encoding a ubiquitin-Gag fusion protein flanked by ClaI restriction sites. This fragment was inserted into the unique ClaI site of the VEE replicon plasmid pVR2 (Pushko et al., 1997) to give pVR2/Ub-Gag. Similar standard PCR protocols were used with appropriate primers to alter the Ub-Gag fusion protein as follows: 1) change the Ub mutant Ala^{76} to either Gly^{76} or Val^{76}, 2) substitute an Arg for the N-terminal Met of Gag, 3) truncate the *gag* sequence at the 3′ end of the capsid region leaving the coding sequence for MA/CA followed by a stop codon, and 4) move the position of ubiquitin to an in-frame insertion between the matrix and capsid

sequences. Sequences for each of these fusion proteins, as well as the nonmyristylated, nonubiquitinated MA/CA region of SIV Gag were inserted individually into the pVR2 replicon plasmid (as diagrammed in Figure 1A).

A second, closely related VEE replicon plasmid, pERK (Kamrud et al., 2007), was used to express mutated forms of MA/CA. The pERK replicon vector differs from pVR2 in that it contains the 3′-most 16 nucleotides of the 5′ UTR of the 26S subgenomic mRNA followed by a unique EcoRV site, and a kanamycin resistance gene in place of the ampicillin resistance gene. In previous work, VEE vectors with and without the 3′-most 16 nucleotides of the 5′ UTR were engineered to express the identical MA/CA sequence and shown to give equivalent protein levels in primary mouse embryo fibroblasts (Cecil et al., 2007). The nonmyristylated MA/CA sequence was amplified using PCR with primers designed to add a downstream stop codon and modified termini to allow insertion into the pERK vector between the unique EcoRV and AscI sites to give the plasmid pERK-MA/CA. The same MA/CA PCR product was cloned into a PCR cloning plasmid to use as template for further mutagenesis using the Quick-Change strategy of mutagenesis (Stratagene). First, three nucleotide changes were introduced at position 1154–1157 (numbering from the 5′ end of the SIVsmH-4i genome; TGG to GAC, codon 36 of MA), changing one codon from Tyr to Asp. This amino acid is located in helix2 of the MA globular domain (Massiah et al., 1994). A clone with this mutation was then used as the template for a second round of mutagenesis in which three nucleotides were changed from TGG to GAC at position 1517–1519, which changed the CA codon 23 from Tyr to Asp. This residue is located in helix I of the N-terminal domain of CA. Trp23Ala (helix I) and Phe40Ala (helix II) have been reported to disrupt CA structure and prevent proper maturation of virus cores (Tang et al., 2001; Tang et al., 2003). Finally, another three nucleotide changes were introduced at position 1990–1992, which generated another Tyr to Asp mutation in CA (HXB2 CA position 184). This residue is located at the dimer interface of the CA C-terminal domain. Trp184 is part of the hydrophobic core of the dimer interface (Gitti et al., 1996; Momany et al., 1996), and a Trp184Ala mutation had been reported to abolish CA dimerization (Mateu, 2002; von Schwedler et al., 2003). In all, a total of three codons were changed in the MA/CA coding domain. The mutated MA/CA was cloned into the pERK vector following the same strategy as above. The new plasmid was designated pERK-ufMACA.

2.3. Production and Titration of VEE Replicon Particles (VRPs)

Plasmid DNA was prepared with the Qiagen HiSpeed Plasmid Maxi Kit. DNA was linearized with Not I, and RNA transcribed *in vitro* using the Ambion mMESSAGE mMACHINE T7 kit. BHK cells were electroporated with a mixture of three RNAs: replicon RNA, capsid helper RNA and glycoprotein helper RNA (Pushko et al., 1997) using a BioRAD GenePulser. Cells were pulsed three times at 850 volts, 25-μF capacitance. Harvest and purification of VRP by sedimentation through a cushion of sucrose were performed as described previously (Davis et al., 2000). VRPs were titrated by infection of BHK cells with serial dilutions of purified VRPs for 16 h at 37°C followed by a 10 min methanol fixation at 4 °C. Cells were incubated with serum from mice vaccinated with null VRP, which express the VEE replicase proteins but no foreign gene. This serum contains antibody to VEE nonstructural proteins present in VRP-infected cells. Cells were washed and incubated with biotinylated anti-mouse IgG followed by strepavidin conjugated to Alexaflour Texas Red. VRP infected cells were scored microscopically for fluorescence under UV illumination.

2.4. Quantitative real-time PCR for VRP genomes

Purified VRP were serially diluted in phosphate-buffered saline (PBS), and several dilutions were extracted using MagMAX Viral RNA Isolation Kit (Ambion) according to manufacturer’s instructions. RNA was treated with the TURBO DNA-free kit (Ambion) to remove any contaminating genomic DNA. Primers and probes for real-time PCR were

designed using Primer Express software (Applied Biosystems). The forward primer V928F (GCCTGTATGGGAAGCCTTCA), the reverse primer V1000R (TCTGTCACTTTGCAGCACAAGAAT), and the 5′FAM and 3′TAMRA labeled probe MEPnsp1 (CCTCGCGGTGCATCGTAGCAGC) (Integrated DNA Technologies) were used to detect a region within the nsp1 gene of the VEE genome. Real-time PCR was performed using the ABI Prism 7000. Real-time PCR reactions were set-up in a total volume of 25 μl including 5 μl RNA, 12.5 μl TaqMan One Step RT-PCR Master Mix (ABI), 900 nM forward primer, 300 nM reverse primer, 250 nM probe, and 0.625 μl MultiScribe Reverse Transcriptase plus RNase Inhibitor. Cycling conditions were 48°C for 30 min, 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in duplicate. The threshold cycle was set manually and copy number for each sample was determined using a standard curve method. RNA transcripts for the standard curve were synthesized from linearized pVR21 plasmid DNA by Ambion mMESSAGE mMACHINE T7 kit. RNA was DNAse treated and lithium chloride precipitated and the concentration was determined by spectrophotometry.

2.5. Pulse-chase labeling of infected cells

BHK cells were plated in 12-well tissue culture dishes at 10^5 cells per well in BHK complete medium. The next day cells were infected with VRPs at an MOI of 5. Cells were incubated for 4 h in complete medium and then washed and incubated in methionine/cysteine-free MEM (MP Biomedicals) with 1% DCS for 1 h. Medium was replaced with 0.5 ml methionine/cysteine free MEM with 1% DCS with or without 10μM lactacystin (Sigma) and incubated for 30 min. Cells were labeled with 50 μCi of ^{35}S-methionine/cysteine per well (Amersham) for 30 min. Label was removed, and cells were washed twice with 0.5 ml of BHK complete medium followed by the addition of 1 ml chase medium (complete BHK media + 4mM unlabeled L-methionine and 4mM L-cysteine, with or without 10μM lactacystin). Cells were either harvested directly (0 h) or incubated for 2, 4, or 6 h (chase). Prior to harvest, cells were washed twice with 1 ml cold PBS. Cells were harvested in 0.25 ml NP-40 lysis buffer (54mM Tris, 170mM NaCl, 13mM EDTA [pH 7.2] + 0.2% igepal [NP-40]). Proteins in cell lysates were separated by SDS-PAGE and analyzed by phosphorimaging (Molecular Dynamics). Half-lives of the heterologous protein expressed from the VRP were determined by regression analysis.

2.6. Immunizations of mice with VRP

VRP were diluted in PBS containing 100 mM calcium and 50 mM magnesium with 1% DCS, and groups of five to six week old BALB/c mice were inoculated subcutaneously in both rear footpads with a total of 10^6 IU unless otherwise noted. Mice were boosted with the same dose four weeks after the primary inoculation. Mice were bled prior to immunization (week 0), prior to boost (week 4), and at weeks 5 and 10.

2.7. Harvest of spleen cells and Elispot assay

Multiscreen 96-well filtration plates (Millipore, ELIIP10SSP) were coated overnight at 4°C with 5μg/ml anti-mouse IFN-γ (Mabtech, AN18). Plates were washed with PBS and blocked with 5% FBS in AIM V medium (Invitrogen). Spleens were harvested and homogenized into single cell suspensions in PBS + 10% Alsever's solution (44.4 mM dextrose, 28.6 mM citric acid, 71.9 mM sodium chloride). Cells were pelleted and red blood cells were lysed by adding 1 ml of sterile water, immediately followed by 10 ml of PBS/Alsever's (PBS/A). Cells were washed twice, then resuspended in RPMI 1640 medium (Invitrogen) with 5% FBS. Splenocytes were overlaid onto a Lympholyte M cushion (Cedarlane) and centrifuged for 30 min at 2500 rpm at room temperature. The buffy coat was removed, resuspended in PBS/A and washed twice. After the last wash, cells were resuspended in AIM V medium, counted and adjusted to 1×10^7 cells/ml and 50 μl were plated into wells of anti-IFN-γ antibody coated 96 well plates

containing either 50 μl of peptide(s) to be tested, an irrelevant control influenza virus hemagglutinin (HA) peptide (IYSTVASSL), or a no peptide control. A pool of three 15-mer SIV Gag peptides shown previously to be reactive with splenocytes from SIV Gag immunized BALB/c mice were used at 2 μg/ml (Cat. #5236, Cat. #5276 and Cat. #5285; NIH AIDS Repository; Cecil et al, 2007). The plates were incubated for 18–24 hours at 37°C with 5% CO_2. Plates were washed with chilled distilled water, incubated on ice for 10 minutes, washed 10 times with wash buffer (PBS with 0.01% Tween-20) and then incubated with 1μg/ml of biotinylated antibody to mouse IFN-γ(Mabtech, R4-64A2,) in PBS containing 0.01% Tween-20, 1% bovine serum albumin (BSA) for 16–18 hours at 4°C. Plates were washed ten times with wash buffer. Strepavidin-alkaline phosphatase (Mabtech) was diluted 1:1000 in PBS/0.01% Tween-20/1% BSA, 100μl was added to each well, and plates were incubated for 1–2 hours at room temperature. Plates were washed 10 times, and spots were developed with 100 μl of BCIP/NBT substrate (Promega) prepared according to the manufacturer's instructions. Spots were counted using an automated plate reader with ImmunoSpot software (Cellular Technology Ltd.).

2.8. Serum IgG ELISA

His-tagged MA/CA protein was made in *Escherichia coli* strain BL21DE3 using the pET24a expression plasmid. Ninety-six well plates (Immulon-4, Thermo Electron) were coated with 200 ng/well of MA/CA-his protein in carbonate buffer (15 mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Sera were diluted in PBS with 0.05% Tween and 10% Sigmablock (Sigma) and added to plates for 2h at room temperature. Plates were washed and incubated with horseradish peroxidase-linked anti-mouse IgG (Sigma) for 2h. Plates were washed and then developed with o-Phenylenediamine substrate (Sigma) for 30 min, followed by an acid-stop with 0.1 M sodium fluoride. Titers represent the serum dilution that corresponded to half the maximum absorbance for each sample measured at 450 nm.

2.9. Statistical analysis of ELISPOT and ELISA results

The Wilcoxon rank-sum test (using Van der Waerden normal scores) was used for our numerous two-group comparisons. The Bonferroni method was used to adjust p-values to account for multiple comparisons. Statistical analyses were performed with SAS statistical software, Version 9.1, SAS Institute Inc., Cary, NC.

RESULTS

3.1. Targeting SIV MA/CA for rapid degradation

Initially, two strategies were used to target MA/CA for rapid degradation, Ub fusion and the N-end rule (Figure 1A). Ubiquitin fusion was achieved by using a mutant ubiquitin with Val at codon 76 to reduce the efficiency of removal of the Ub monomer from the N-terminus of the fusion protein (Johnson et al., 1992) and linking the Ub coding sequence in frame to the MA/CA coding sequence in the VEE vector (Ub-V-MA/CA). In a second configuration, the sequence encoding Ub-Val^{76} was introduced in-frame between the sequences encoding Matrix and Capsid (MA-UbV-CA). In both of these contexts, the non-removable Ub would serve as a substrate for rapid polyubiquitination and would target the protein for subsequent proteasomal degradation. Another strategy involved the N-end rule, which describes the correlation between the half-life of a protein and the identity of the N-terminal amino acid. Ub fusion constructs in which the efficiently recognized Ub-Gly^{76} cleavage site was retained allowed the original initiating methionine codon of MA/CA to be changed. Upon Ub cleavage, the new N-terminal residue of MA/CA was exposed. This approach was used to make MA/CA with a destabilizing arginine N-terminal residue (Ub-R-MA/CA). The N-recognin/E2 complex would recognize the arginine and mark an internal lysine for ubiquitination (Meinnel, Serero, and Giglione,

2006; Varshavsky, 1996). The polyubiquitinated protein would then enter the proteasomal degradation pathway.

Pulse-chase experiments demonstrated that each of the MA/CA proteins engineered for rapid degradation had a shorter half-life in cultured cells than the unmodified MA/CA **(**Figure 1B**)**. The N-end rule version of MA/CA, Ub-R-MA/CA, was the most rapidly degraded with a $t_{1/2}$ of 40 min compared with the native MA/CA (>360 min). The Ub fusion proteins, Ub-V-MA/CA and MA-UbV-CA, also had substantially decreased half-lives of 77 min and 100 min, respectively. Both Ub fusion proteins were slightly larger than the proteins produced by MA/CA and Ub-R-MA/CA VRP due to their covalent linkage to the 76-residue Ub molecule. This is evident in Figure 1B, as the bands migrate slightly slower for Ub-V-MA/CA and MA-UbV-CA. A faint lower band corresponding to the size of MA/CA is also visible with Ub-V-MA/CA and MA-UbV-CA, which may represent some leakiness of the Val^{76} cleavage site. In the presence of the proteasome inhibitor lactacystin, degradation kinetics for all of the constructs were similar to unmodified MA/CA, indicating that the degradation of the N-end rule and Ub fusion forms of MA/CA was proteasome dependent (data not shown).

3.2. Cellular immune response to rapidly degraded MA/CA antigen in vivo

A series of reports describes the effect of targeting antigens for rapid degradation on the immune response using various vaccine vectors. Initial experiments were performed to determine what effect a rapidly degraded antigen would have on the immune response when expressed from a VRP vector packaged with a mutant glycoprotein spike (3014). The 3014 spike attenuates virulence in the context of VEE virus and has been used as a safety feature in VRP vaccines produced for pre-clinical and clinical trials. Mice were vaccinated with 10^6 infectious units (IU) of 3014-VRP expressing MA/CA, Ub-R-MA/CA or Ub-V-MA/CA. Four weeks later mice were boosted with the same dose. At six weeks after the boost mice were sacrificed, spleens were harvested, and purified splenocytes were assayed by IFNγ-ELISPOT. For each mouse, the median number of spots formed by splenocytes stimulated with a pool of three SIV Gag peptides (positive pool) less the average number of spots formed by splenocytes incubated with an irrelevant peptide or no peptide was plotted as spots per million cells in Figure 2A. Mice vaccinated with unstable forms of MA/CA (Ub-R-MA/CA or Ub-V-MA/CA) displayed a significantly reduced cellular immune response in comparison to mice vaccinated with unmodified MA/CA (p=0.01 and p=0.01, respectively). Decreasing stability of MA/CA also correlated with decreased serum antibody responses at one week and six weeks after boost measured by ELISA (data not shown).

3.3. Altered immune responses to rapidly degraded MA/CA antigen in vivo when administered in the context of the wild-type glycoprotein coat

VRP particles packaged with different glycoprotein coats may target different cells *in vivo,* and the wild type spike (GP3000) targets to Langerhans-like dendritic cells, which then begin to display surface markers of professional antigen-presenting cells (MacDonald and Johnston, 2000). Based on these findings, the immunogenicity of the ubiquitin-linked MA/CA expressing VRP when packaged using a glycoprotein helper RNA encoding the wild-type VEE glycoprotein coat (VRP-3000) was assessed. In a mouse experiment with the same dose and time line as the first experiment, Ub-V-MA/CA and MA-UbV-CA VRP still induced a weak cellular immune response in comparison to the unmodified MA/CA VRP, but VRP expressing MA/CA with a destabilizing N-terminus, Ub-R-MA/CA, produced a response equivalent to MA/CA (p-value did not reach statistical significance; Figure 2B). A second experiment directly comparing MA/CA and Ub-R-MA/CA delivered by VRP-3014 or VRP-3000 at an identical dose of 10^6 IU per mouse is described in Figure 2C. As in the first two experiments, expression of the rapidly degraded Ub-R-MA/CA gave fewer IFNγ-expressing cells compared to unaltered MA/CA-VRP in the 3014 coat, but a similar response when packaged with the

3000 coat. Interestingly, the overall number of IFNγ producing cells was higher, with borderline significance, for unmodified MA/CA when given in the 3000 coat compared to the 3014 coat (unadjusted p=0.048).

3.4. VRP titer determination by real-time PCR

The V3014 mutant was isolated following stringent selection for entry into cultured BHK cells (Johnston and Smith, 1988). Further studies demonstrated that VRP-3014, but not VRP-3000, use heparan sulfate to bind to BHK cells in culture (Bernard, Klimstra, and Johnston, 2000). Based on this observation it was predicted that VRP-3014 would be more efficient at infecting BHK cells than VRP-3000, and that their specific infectivities (particle/IU) measured on BHK cells might differ. On the other hand, efficiency of infection of target cells *in vivo* may be governed by very different parameters than infection of BHK cells. Therefore, to accurately compare the immune responses induced by VRP packaged with V3014 or V3000 glycoproteins, the titer of the VRP preparations was determined by an unbiased method. A quantitative real-time PCR assay was developed to quantify VRP genomes in samples of RNA extracted from purified VRP preparations. Titers were calculated in genomes/ml and compared with the BHK IU/ml titers (Table 1). Replicons packaged in the 3014 glycoproteins gave similar real-time PCR titers compared to the BHK infectivity titers, indicating a high efficiency of infection of BHK cells. Replicons packaged in the 3000 coat, however, had genome titers approximately 100-fold higher than their corresponding BHK titers, signifying that VRP-3000 have a lower efficiency of infection in BHKs. In retrospect, the comparison of immune responses shown in Figure 4 was based on groups of mice receiving either approximately 10^6 particles of VRP-3014 or approximately 10^8 particles of VRP-3000. This difference in dose may explain the greater number of MA/CA-specific IFNγ-secreting cells induced by MA/CA-expressing VRP-3000 than MA/CA-expressing VRP-3014 (Figure 2C).

Using the titers obtained from the real-time PCR assays, groups of mice were inoculated with 10^6 genome equivalents of VRP expressing stable or unstable forms of MA/CA packaged with either the 3014 or the 3000 spike (Figure 3). The numbers of MA/CA-specific splenocytes, measured by IFNγ ELISPOT, were not statistically different for unmodified MA/CA regardless of coat. However, the rapidly degraded form of MA/CA was again associated with a decreased response compared to stable MA/CA in the context of the 3014 spike (p=0.04). The immunogenicity of the rapidly degraded Ub-R-MA/CA can be compared in two different coats, and produced a significantly better response in the 3000 coat than when given in the 3014 coat (p=0.009). Taken together, this suggests that the two glycoprotein coats target functionally different cells.

When the dosage of VRP was adjusted for genome equivalents, the absolute number of BHK-infectious units was decreased approximately 100-fold for the 3000-VRP groups. Interestingly, the decrease in dose did not produce a corresponding decrease in the immune responses in the 3000-VRP vaccinated groups. It is possible that the number of target cells for the VRP-3000 is limiting, and are all infected by the lower dose. An increased dose cannot therefore give a proportional increase in the immune response.

Figure 4A and 4B show the median antibody titers of the mice from this experiment at week 4 and week 10. The antibody titers to MA/CA were significantly decreased in the Ub-R-MA/CA group compared to the MA/CA group with 3014-VRP, but not 3000-VRP, at both 4 weeks (pre-boost, p=0.00003) and 10 weeks (6 weeks after boost, p=0.001). These results reflect the trends observed in the ELISPOT experiments, and indicate that both the cellular and humoral responses to long-lived or short half-life immunogens are affected similarly when delivered by VRP-3014 or VRP-3000.

3.5. Mutations which cause misfolding and subsequent degradation do not affect the immunogenicity of MA/CA in the wild-type glycoprotein coat

Mutations which disrupt protein folding were introduced into MA/CA (ufMA/CA) as another strategy to induce rapid antigen degradation. In pulse-chase experiments this form of MA/CA was degraded with similar kinetics and to the same extent as Ub-R-MA/CA ($t_{1/2}$= 55 min). However, in contrast to Ub-R-MA/CA, the presence of the proteasome inhibitor lactacystin did not prevent the degradation of ufMA/CA, suggesting that degradation of this protein may occur in a proteasome-independent manner. The characteristics of the unfolding mutant provided an opportunity to examine the effect of another, perhaps functionally different, antigen on the VRP-induced immune response. ufMA/CA was compared to unmodified MA/CA and Ub-R-MA/CA delivered by VRP-3000 (Figure 5). The MA/CA specific cellular response, measured by IFNγ ELISPOT, was not statistically different for any of the groups at 6 weeks after boost, indicating that rapid antigen degradation caused by misfolding, like that caused by the N-terminal Arg, does not result in the loss of a cellular immune response when delivered by VRP with the wild type glycoprotein spike.

DISCUSSION

Several viruses are being modified and tested as vaccine vectors, including VEE. The properties of VEE that would be desirable in a vaccine vector include lack of anti-VEE immunity in most humans, replication in the draining lymph node, known attenuating mutations, and induction of mucosal immune responses (Davis et al., 1994; Grieder et al., 1995; MacDonald and Johnston, 2000; Thompson et al., 2006). In this study a VEE derived vaccine vector, VRP, was characterized by examining the effect of rapid antigen degradation on immunogenicity induced by VRP with different glycoprotein coats. Forms of SIV MA/CA with significantly reduced half-lives were made using several strategies for targeted antigen degradation, including ubiquitin fusion, the N-end rule, and destabilizing mutations. When compared to unmodified MA/CA, the rapidly degraded forms of MA/CA resulted in a significantly reduced cellular immune response in vaccinated mice when delivered by VRP carrying the attenuated 3014 glycoprotein spike. This result is consistent with similar studies using another alphavirus vector, Semliki Forest Virus (rSFV), expressing a rapidly degraded form of influenza nucleoprotein (NP) (Huckriede et al., 2004). As was found with VRP-3014, the unstable ubiquitin-NP fusion protein produced poor immune responses in mice compared with the unmodified antigen, an outcome attributed to the lack of dendritic cell infection seen with the rSFV vector. These results with two alphavirus vectors are in contrast to several previous studies, which used primarily DNA or recombinant vaccinia virus vectors and reported successful enhancement of cellular immune responses by targeted antigen degradation.

When VRP were packaged with the wild type V3000 spike, however, the correlation between rapid degradation of Ub-R-MA/CA and poor immune response did not hold. In experiments comparing equal IU doses of VRP expressing MA/CA and Ub-R-MA/CA, the response to the rapidly degraded antigen was equal or slightly increased compared to the unmodified antigen when VRP carried the V3000 spike, but decreased with VRP-3014 vaccination. Furthermore, a misfolded mutant of MA/CA, which is also rapidly degraded, was able to induce a comparable immune response to MA/CA and Ub-R-MA/CA when delivered by VRP-3000. When these results are viewed in the context of previous work done on the *in vivo* cell targeting of the VEE glycoprotein coats (MacDonald and Johnston, 2000), it seems likely that the difference in the effect of antigen stability observed here is due to functional differences in the target cells. As demonstrated previously (Norbury et al., 2004), rapid antigen degradation has a detrimental effect on immunogenicity in the context of cross-presentation, but not direct presentation. In fact, the ideal substrates for cross priming were stably expressed whole proteins. Based on these findings and the phenotypic differences observed for VRP-3000 and VRP-3014 in this

and previous work, we suggest that the cells targeted by the wild type and mutant glycoproteins differ in their antigen presentation capabilities. Specifically, it appears that VRP-3014 infected cells induce immune responses by cross-presentation of antigen, which would account for the loss of immunogenicity when VRP-3014 expressed a rapidly degraded antigen.

If previous findings suggesting that V3000 infects an APC are correct, then it could be predicted that a rapidly degraded antigen delivered by VRP-3000 would enhance the response over that induced by the stable form of the antigen. In one experiment, a slight increase in immune response was correlated with rapid antigen degradation, but this difference did not reach statistical significance. In the second experiment, the number of MA/CA-specific IFNγ-secreting splenocytes was not significantly different for the stable and the unstable forms of antigen. Therefore, it appears that the supply of peptides generated from the high level protein expression directed by the VRP genome is able to saturate a limiting number of MHC I complexes on the VRP-3000-infected APC, and that increasing the efficiency of protein processing does not increase the level of presentation.

Unexpectedly, the Ub fusion proteins Ub-V-MA/CA and MA-UbV-CA differed from the N-end rule protein Ub-R-MA/CA when delivered by VRP-3000. While these proteins were also short-lived *in vitro*, they did not behave similarly to Ub-R-MA/CA *in vivo*, and were less immunogenic than stable MA/CA in both VRP-3014 and VRP-3000. These two Ub fusion forms of MA/CA were destabilized by a different strategy than Ub-R-MA/CA, which may explain the difference in outcome. The mechanisms of entry into the Ub-proteasome pathway are different for N-end rule proteins and Ub-fusion proteins, which could potentially affect the efficiency of processing. It is possible, although purely speculative, that compartmentalized pathways for processing of peptides in the VRP-3000 targeted cells work with different efficiencies on proteasome substrates coming from the two rapid degradation pathways, while these pathways are equivalent in the VRP-3014 targeted cells.

Studies to characterize the *in vivo* target cells for VRP enveloped in the two different spikes are ongoing, and results obtained to date suggest that the V3000 glycoprotein spike interacts with immature dendritic cells both *in vivo* (MacDonald and Johnston, 2000) and *in vitro* (Moran et al., 2005). Cell surface markers on VRP-3000 infected cells in the draining lymph node indicated that the dendritic cells undergo only partial maturation following VRP infection (MacDonald and Johnston, 2000). Increasing the dose of VRP-3000 allowed detection of infected cells with surface markers found on mature DCs and macrophages. In vitro, VRP-3000 infected bone marrow-derived dendritic cells display surface markers expected of mature dendritic cells (CD80, CD86 and CD83), but also secrete a constellation of pro-inflammatory cytokines (IFNα, TNFα, IL-6 and IL-12p70) that are not secreted by mature DCs (Moran et al., 2005). Therefore, VRP-3000 infection appears to alter the maturation program of these cells, giving them unique properties. *In vitro* infection of dendritic cell subsets with VRP-3014 has been reported recently (Nishimoto et al, 2007). However, until a direct comparison of VRP-3000 and VRP-3014 infection of dendritic cells *in vitro* is performed, it remains to be seen whether distinct DC subsets of these cultured primary cells are targeted by wild type and mutant glycoproteins. As further work both *in vivo* and *in vitro* identifies the distinct cellular targets for VRP-3000 and VRP-3014, differential viral effects on protein degradation and peptide presentation pathways also may be discovered.

In this study the effects of specific infectivity differences between VRP-3014 and VRP-3000 on their comparative immunogenicity also were examined. Evidence that VRP packaged in V3014 glycoproteins infect BHK cells more efficiently than VRP-3000 suggested that titers based on BHK infectious units may represent different numbers of particles in each case. Determination of VRP titer by real-time PCR confirmed this idea, and titers measured by BHK infectivity underestimated the concentration of V3000 VRP by approximately two logs.

However, the comparison of VRP expressing either the unmodified MA/CA or Ub-R-MA/CA in the two coats using a dose based on an equal number of genomes, instead of BHK IU, showed the same trends in the effect of glycoprotein spike on immunogenicity of stable and unstable MA/CA. In addition, the differences in the magnitude of the responses to the unmodified MA/CA in the two coats were eliminated when the *in vivo* dose was adjusted based on genome quantitation. This indicates that the different effects of antigen stability seen with VRP-3000 and VRP-3014 are not due merely to quantitative differences in infectious *in vivo* dose.

While the rapid antigen degradation approach has been used with other vaccine vectors as a strategy to increase cellular immune responses, it has consistently resulted in a substantial corresponding loss of humoral responses (Andersson and Barry, 2004; Delogu et al., 2000; Fu et al., 1998; Rodriguez, Zhang, and Whitton, 1997; Wu and Kipps, 1997). In this study, the antibody responses to the VRP-delivered rapidly degraded antigens mirrored the cellular response in all groups. Based on previous studies of rapidly degraded antigens, it was predicted that Ub-R-MA/CA would induce minimal antibody production, regardless of glycoprotein coat, due to the rapid and complete degradation observed *in vitro*. Surprisingly, in the 3000 coat, Ub-R-MA/CA retained the ability to induce an antibody response. This suggests that the cell(s) targeted by VRP-3000 may contribute to the humoral immune response, at least in part, by presenting peptides on MHC II, in which case rapid degradation would not be detrimental.

VRP vaccine vectors are capable of stimulating robust cellular and humoral immune responses *in vivo*, and in this report we show that functional differences in the cellular targets of VRP packaged in a mutant or wild type glycoprotein coats can significantly influence the magnitude of these immune responses. This suggests that optimization of these vectors for immunization may involve, at a minimum, integrated evaluation of both vector and immunogen configuration.

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Figure 1A

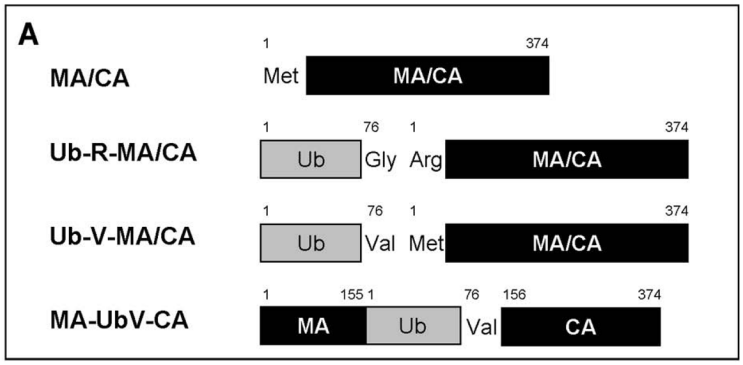

Figure 1B

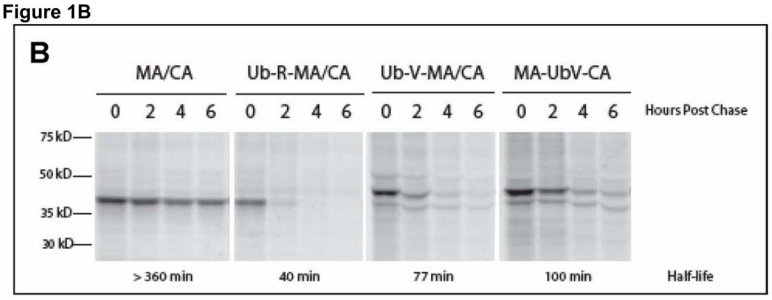

Figure 1.

(A) Diagram of VRP protein products used in this study. (B) Pulse-chase analysis of VRP infected cells. VRP infected BHK cells were starved for methionine and cysteine from 4 to 5 hpi and pulse labeled with ^{35}S-Met and ^{35}S-Cys for 30 min and then chased with an excess of unlabeled Met and Cys for 0h, 2h, 4h or 6h. Cell lysates were separated by SDS-PAGE and bands were quantitated by phosphorimager.

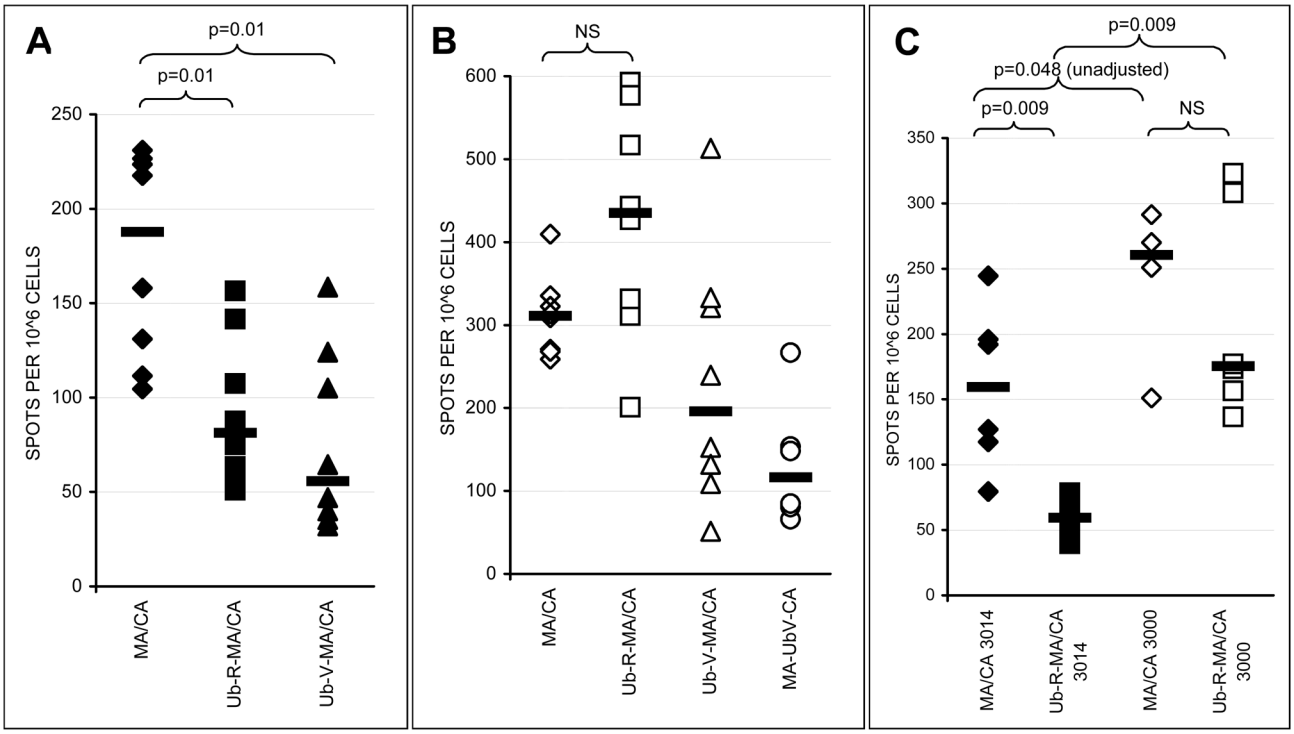

Figure 2.

Groups of BALB/c mice were immunized subcutaneously in the rear footpads with 10^6 IU of VRP. At week 10 mice were sacrificed and spleens removed. Splenocytes were purified and IFN-γ secreting cells were detected by ELISPOT assay in the presence of a pool of SIV Gag peptides. Each data point represents spot forming cells per 10^6 cells for an individual mouse, and bars represent the median values. (A) ELISPOT assay at 10 weeks on spleen cells from BALB/c mice immunized with 10^6 IU of 3014-VRP expressing MA/CA (◆), Ub-R-MA/CA (■) or Ub-V-MA/CA (▲). (B) ELISPOT assay at 10 weeks on spleen cells from BALB/c mice immunized with 10^6 IU of 3000-VRP expressing MA/CA (◇), Ub-R-MA/CA (□), Ub-V-MA/CA (△) or MA-UbV-CA (○). (C) ELISPOT assay at 10 weeks on spleen cells from BALB/c mice immunized with 10^6 IU of 3014-VRP expressing MA/CA (◆) or Ub-R-MA/CA (■), or 3000-VRP expressing MA/CA (◇) or Ub-R-MA/CA (□).

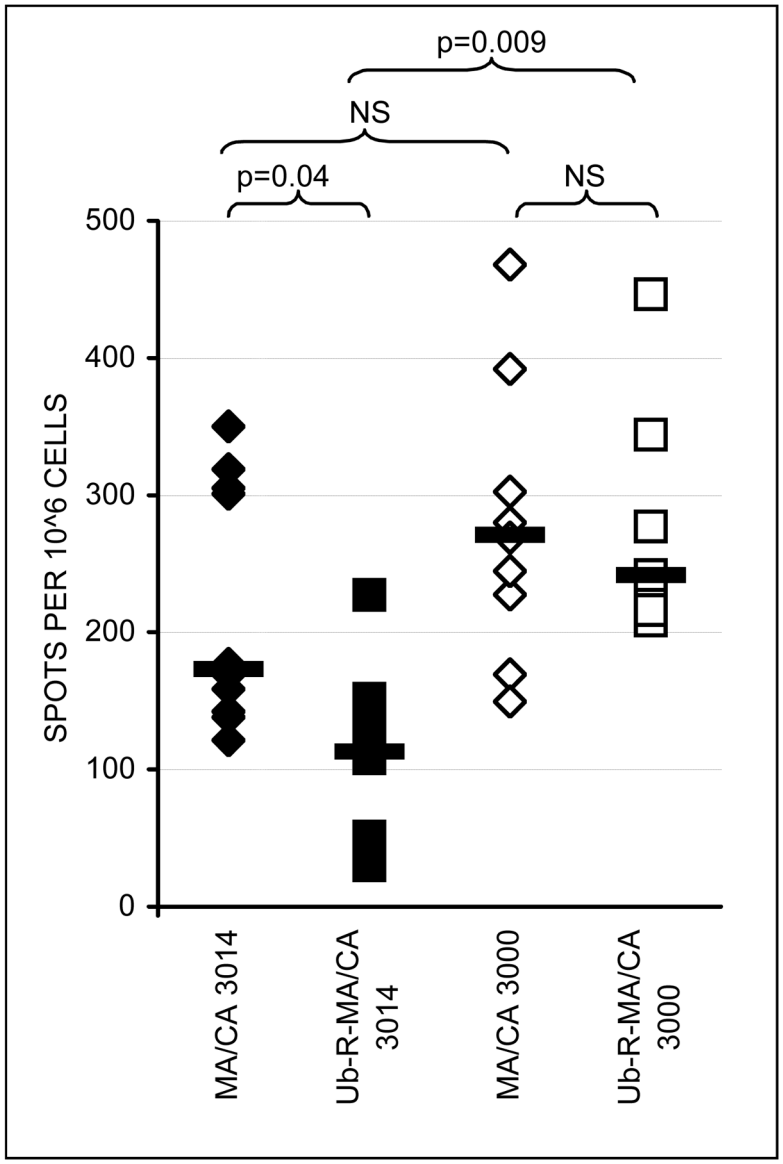

Figure 3.

ELISPOT assay at 10 weeks on spleen cells from BALB/c mice immunized with 10^6 genome equivalents of 3014-VRP expressing MA/CA (◆) or Ub-R-MA/CA (■), or 3000-VRP expressing MA/CA (◇) or Ub-R-MA/CA (□). Each data point represents spot forming cells per 10^6 cells for an individual mouse, and bars represent the median values.

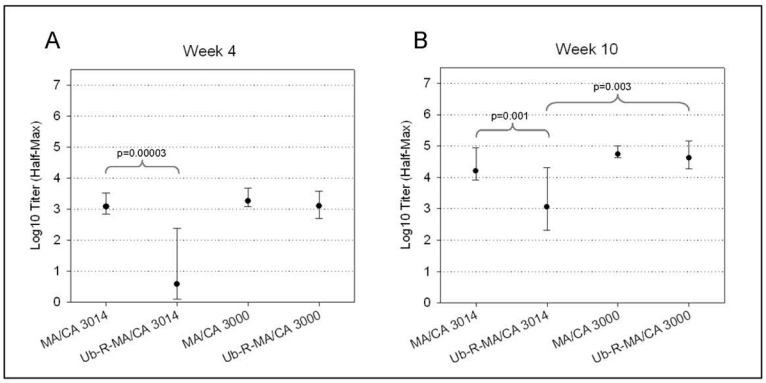

Figure 4.

Serum IgG titers from mice immunized with 10^6 genomes of MA/CA or Ub-R-MA/CA in either the 3014 or 3000 glycoprotein coat at week 4 (A, pre-boost) and week 10 (B, 6 weeks post-boost) after immunization. Points represent the median value of Log10 titers at half the maximum absorbance for each sample, and error bars represent the range of values for each group.

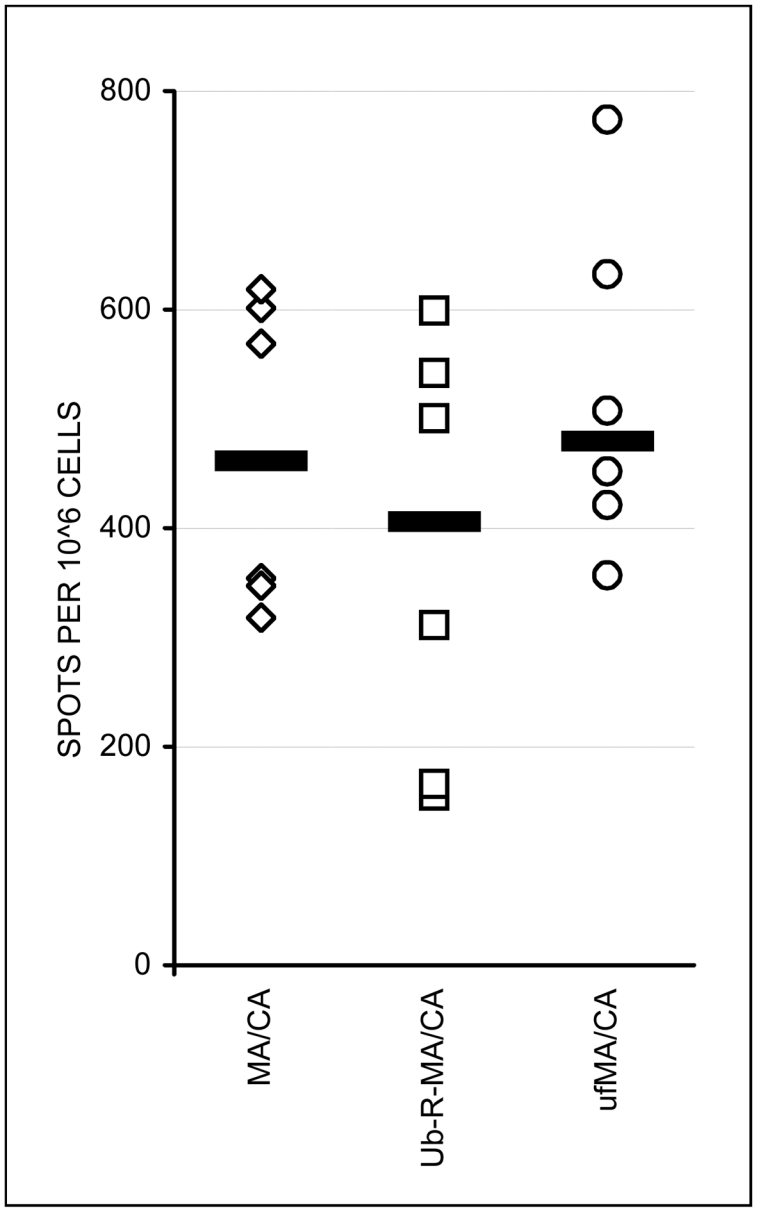

Figure 5.

Immunogenicity of MA/CA unfolding mutant. ELISPOT assay at 10 weeks on spleen cells from BALB/c mice immunized with 10^6 IU of 3000-VRP expressing MA/CA (◇), Ub-R-MA/CA (□), or ufMA/CA (○). Each data point represents spot forming cells per 10^6 cells for an individual mouse, and bars represent the median values.

Table 1

Real-time PCR titers

Protein Expressed	GP Coat	BHK Titer (IU/ml)	Real-time PCR titer (Genomes/ml)	Genomes per IU
MA/CA	3014	3.11×10^{9}	2.76×10^{10}	8.9
MA/CA	3000	6.32×10^{7}	5.35×10^{9}	84.6
Ub-R- MA/CA	3014	5.44×10^{9}	1.92×10^{10}	3.5
Ub-R- MA/CA	3000	1.04×10^{8}	1.33×10^{10}	127.9